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Dissociation of the Hepatic Phenotype from HNF4 and HNF1α Expression

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Dedifferentiated cells have served as tools to understand the molecular consequences of the loss of tissue-specific pathways. Here we report the characterization of one of these cell lines, M29, which lacks the liver-enriched HNF4-HNF1α pathway, in order to determine if this class of variant cell lines could provide additional information regarding requirements for tissue-type expression. We report that although the liver-specific α1-antitrypsin (α1AT) gene remains silent despite reactivation of the HNF4/HNF1α pathway in the M29 cells, the frequency of activation of an integrated α1AT-APRT transgene is increased 1000-fold in response to these transcription factors. The human α1AT locus (introduced via chromosome transfer) also remained silent on these cells, despite HNF4 and HNF1α expression. Results from cell fusion experiments suggest that the defect in the M29 cells is recessive. Results suggest that the M29 cells contain a defect that represses liver gene expression despite the presence of the HNF4/HNF1α pathway.

KEY WORDS: Hepatocyte nuclear factor1; hepatocyte nuclear factor 4; hepatoma; alpha-1; antitrypsin; gene silencing.

INTRODUCTION

The role of chromatin remodeling in gene activation has only recently been appreciated. Many studies have clarified a role of modification of histones (acetylation, methylation and phosphorylation) and DNA (methylation) in regulation of chromatin [1]. Although much is understood with regard to activation of genes that are already active (enhanced transcription), significant questions still remain regarding the mechanism of activation of previously silent genes in the genome (such as those spacially and temporally activated during differentiation and development). Several studies in a variety of tissue culture models have shown that the mere presence of transcription factors alone is not sufficient to activate tissue-specific genes [2–8].

Hepatocyte nuclear factor-lα (HNF1α) and –4 (HNF4) are components of a liver-enriched transcriptional activation pathway. This pathway is considered to play an important role in establishment and maintenance of the hepatic phenotype [9–13]. Both HNF4 and HNF1α function via binding to upstream DNA sequences of target

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genes. Loss of these factors in dedifferentiated hepatoma cells results in silencing of expression of a large panel of liver-specific genes [14, 15]. Further evidence for the importance of these factors in hepatic gene regulation comes from the identification of functionally important binding sites in promoters of a large number of liver genes [16]. Recently, Young and collaborators reported that HNF1α binds the promoters of over 200 liver-specific and pancreas-specific genes *in vivo* [13]. HNF4$^{-/-}$ mice die early in embryogenesis, suggesting that HNF4 plays an essential role in early development [17]. More recently, HNF1α has been also shown to be functionally important in β-cell development and in the transcription of β-cell–specific genes [9, 10, 13, 18–20]. Indeed, mutations in both HNF1α and HNF4 genes have been shown to map to disease loci of a rare subtype of type II diabetes called Maturity Onset Diabetes of the Young (MODY) [21]. Genetic knock-out experiments of HNF1α in mouse models have exhibited type II diabetes symptoms [20, 22].

Molecular details of HNF4/HNF1α pathway activation have been investigated in several cell culture systems. HNF4 and HNF1α are members of a regulatory loop, in which each member activates transcription of the other [23, 24]. Autoregulation of HNF1α has also been reported [25, 26]. Interestingly, HNF4 expression precedes HNF1α expression during mouse development [27, 28], suggesting that HNF4 is a key player in initiating pathways leading to liver-specific gene expression.

Cultured hepatoma cell lines and their dedifferentiated derivatives have been extensively used to determine mechanisms of gene repression and activation of liver – specific genes. It is striking that predictions resulting from these cell culture models conflict with results using transgenic knock-out mice. Specifically, it has been shown that introduction of HNF4 and/or HNF1α into dedifferentiated hepatoma cells generally rescues expression of the endogenous HNF4 and HNF1α genes as well as downstream target liver-specific genes [24, 29–32].

Likewise, hepatoma X fibroblast cell hybrids also show loss of liver expression (including absence of HNF4 and HNF1α). However, introduction of these transcription factors into the hybrids generally fails to restore liver-specific gene expression [7, 8, 33], although HNF4 introduction was shown to rescue endogenous HNF1α expression in such hybrids [8]. Unlike karyotypically complete whole cell hybrids, Bailly et al. [32] showed that the introduction of either HNF4 or HNF1α in cell hybrids with reduced chromosome content resulted in cross-activation of the corresponding endogenous gene as well as other hepatic functions.

Taken together, results from both dedifferentiated hepatoma cell lines and from hepatoma x fibroblast hybrid cells suggest that HNF4 and HNF1α play a crucial role in activation of liver gene expression. However, mice lacking HNF4 continue to express HNF1α and HNF1α-deficient mice continue to express HNF4 [28, 34]. Indeed, the expression of the majority of liver genes silenced in dedifferentiated cells and cell hybrids are unaffected or are only mildly affected by the absence of either HNF4 or HNF1α in transgenic mice in liver [28, 34] or pancreatic cells [20].

To further analyze the link between HNF4 and HNF1α and liver gene expression, we have reported the characterization of hepatoma -derived cell lines which were selected for failure to drive a selectable APRT transgene under the control of the human alpha−1 antitrypsin (α1AT) promoter (a promoter dependent on HNF4 and HNF1α) [7, 35–37]. Most of these hepatoma variant cell lines fail to express HNF4 and HNF1α as well as most liver-specific genes, yet can be rescued by the introduction of

HNF4 and/or HNF1α transgenes [29, 31]. However, certain hepatoma variant cell lines could not be rescued by the introduction of HNF4 or HNF1α.

Here we characterize the M29 cell line, a hepatoma variant cell line which lacks HNF4 and HNF1α, but in which the introduction of these factors fails to rescue hepatic gene expression [29]. The M29 cells failed to express 7 of 8 liver-specific genes tested, yet clones that survived selection against the α1AT-APRT transgene expression show complete rescue of liver gene expression. We show that although HNF4 rescues HNF1α expression, the endogenous α1AT gene remains silent. We show that HNF4 and HNF1α are able to bind DNA and result in a 1000–fold increase in the reversion frequency to $APRT^+$. An introduced human α1AT locus was also refractory to reactivation by HNF4 and HNF1α. These results suggest that a repressor-like activity is present in the M29 cells that prevents HNF4 and HNF1α from activating the endogenous α1AT promoter, but that the α1AT-APRT transgene located in a context that located free from the observed repression. Possible ramifications of these results are discussed.

MATERIALS AND METHODS

Cell Lines and Conditions

All cell lines described are derived from the rat liver tumor line H4IIEC3. Fg14 cells are an adenine phosphoribosyltransferase-positive ($APRT^+$), xanthine-guanine phosphoribosyltransferase-positive (GPT^+) cell line derived from the $APRT^-$ and hypoxanthine-guanine phosphoribosyltransferase-negative ($HPRT^-$) Fado–2 cells by stable transfection of *gpt* and *aprt* transgenes driven by the human α1-antitrypisin (α1AT) gene promoter (–640 to –2 bp) [35]. Dedifferentiated cells lines, including M29 cells, were derived from the Fg14 cells by negative selection against both APRT and GPT transgene gene expression using 20 μg/ml 2,6-diaminopurine (DAP) and 30 μg/ml 6-thioxanthine, respectively [35]. M29 cells have a reversion rate to $APRT^+$ of approximately 10^{-5}. All cells were grown and expanded in 1:1 Ham's F−12-Dulbecco's modified Eagle's medium plus 5% fetal bovine serum (Gibco) and penicillin plus streptomycin.

RNA Analysis

Cytoplasmic RNA was extracted from nearly confluent cell monolayers as previously described [31]. RNA (5 μg) was extracted by NP-40 lysis, size fractionated on 1% agarose−2.2 M formaldehyde gels (Bulla et al., 1992), and transferred onto a nylon filter. The filters were prehybridized and probed at 42°C in 50% formamide, 5X SSPE (1X SSPE = 150 mM NaCl, 1 mM EDTA, 10 mM sodium phosphate, pH 7.4), 1% SDS, 5 × Denhardt's solution (1 × Denhardt's = 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% BSA) and 10 μg/ml each poly-A and poly-C (Pharmacia). Filters were hybridized to specific DNA probes labeled with ^{32}P-dCTP by the random hexamer primer method [38] or an α1AT specific 540-nucleotide ^{32}P-UTP-labeled riboprobe from linearized pAT500.2 as previously described [7]. Probe was added in the same hybridization solution, and the filters were incubated overnight at 42 or 65°C (α1AT). Cloned DNA sequences from α-tubulin (Kα−1 [39]), aldolase B

(pHL413 [40]), β-fibrinogen and γ-fibrinogen [41], phosphoenolpyruvate carboxykinase (pPCK−10, [42]), transferrin (plivS−6, [43]), argininosuccinate synthetase (pASr) and argininosuccinate lyase (pALr−3) [44], albumin (pRSA57, [45] and rat HNF1α (3.6 kb cDNA) were used. Probed filters were washed twice for 5 min each in 2 x SSC, 0.1% SDS at RT, then 30 min in 0.2 x SSC, 0.1% SDS at 52°C, and exposed to film for 1–5 days. For the α1AT probe, filters were washed in identical conditions, except that all solutions were at 65°C.

To detect HNF4 expression, a 179 nt riboprobe [29] was used. Total cellular RNA (10 μg) was incubated with 1×10^6 cpm of HNF4 riboprobe and incubated overnight at 52°C. The mixture was digested with RNase T1 + A, and protected fragments were resolved on 8% denaturing polyacrylamide gels. The gels were dried and exposed to film for 1 to 5 days.

Microcell-Mediated Chromosome Transfer

Chromosome donor cell line HDm-5 is a mouse fibroblast cell line containing two copies of human chromosome 14 marked with a neomycin resistance gene [46]. HDm-5 cells were incubated in the presence of 10 μg/ml colcemid for 48 h to micronucleate the cells. Cells were then trypsinized, replated onto plastic bullets and enucleated by centrifugation in the presence of 10 μg/ml cytochalasin B, as previously described [47]. Cells were then centrifuged and resuspended in serum-free medium and filtered through 8- and 5-μm filters. Particles were then concentrated by centrifugation, resuspended and added to a confluent monolayer of M29 cells. The cell mixture was then fused using polyethylene glycol for one min. Cells were rapidly washed three times, then medium added. After 24 h, cells were split 1:20 into selective medium containing 3 mM Ouabain plus 500 μg/ml G418. Individual microcell hybrids were picked and expanded after three weeks using cloning rings. As a control, M29 cells alone were treated under identical conditions.

PCR Reactions

PCR was performed using 200 ng of genomic DNA template, 200 mM dNTPs, 1.5 mM magnesium, 100 ng each primer, and 1.2 U *Taq* polymerase using a Thermolyne thermocycler in a volume of 20 µl. Samples were denatured at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 58°C for 45 s, and 72°C for 60 s. Primer sequences for human α1AT were used to screen for the presence of human chromosomes 14 as described by Theune *et al*. [48]. Amplification products were resolved on 3% agarose gels containing ethidium bromide and photographed.

Induction of NF-κB

Cells were treated in serum-free 1:1 Ham's F12:Dulbecco's modified Eagle's medium containing a combination of the following: 1 μg/ml lipopolysaccharide (LPS), 5 U/ml interleukin−1β (IL−1β), 100 U/ml interferon-γ (IFG-γ), and 50 ng/ml tumor necrosis factor-α (TNF-α) Sigma-Aldrich) as previously described [49].

Nuclear Extracts

Nuclear extracts were prepared according to the method of Schreiber et al. [50] with modifications. Briefly, cells were removed with a cell scraper, centrifuged for 3 min at 550 × g, supernatant was discarded, and the pellet was resuspended in 400 μl of chilled buffer I (5 mM $MgCl_2$, 5 mM β-glycerol phosphate, 0.2 mM EDTA (or EGTA), 0.3 M sucrose, 1 mM DTT, 0.5 mM PMSF, 1 × protease inhibitor cocktail, 20 mM Tris-HCl, pH 7.8). The protease inhibitor cocktail is 6.0 ng/ml leupeptin, 0.1 μg/ml aprotinin, 40 μM benzamidine, and 20 ng/ml antipain. NP-40 was added to a concentration of 0.5% and the samples were incubated on ice for 1 min before being spun for 3 min at 2,200 × g. The supernatant was discarded and the pellet resuspended in 100 μl of chilled buffer II (5 mM $MgCl_2$, 350 mM NaCl, 0.2 mM EDTA, 10 mM β-glycerol phosphate, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF, protease inhibitor cocktail, 10 mM Tris-HCl, pH 7.8). Samples were incubated on ice for 15 minutes and centrifuged for 15 minutes at 4°C at 10,000 × g. The pellet was discarded and the supernatant was transferred to a new tube and stored at −70°C.

Electrophoretic Mobility Shift Assay (EMSA)

Ten μg of nuclear protein were added to 4 mM Hepes, 10 mM NaCl, 0.3 mM $MgCl_2$, 2% glycerol, 0.6–1.2 μg of poly-dIdC, and a 1 × protease inhibitor cocktail described in the nuclear extract procedure. To this solution, 1×10^4 cpm of the labeled oligonucleotide was added and incubated with the protein for 15–30 minutes at 4°C. The following DNA oligonucleotides were used in the binding assays: HNF1α, CCTTGGTTAATATTCACC; HNF4, AGCAAACAGGGGCTAAGT-CCACTGGCTG, NF-κB TCGAGGGCUGGGAUUCCCCATCTC, and Oct−1 GGGGGTAATTTGCATTTCTAAGGG. Dye was added and the samples were subjected to polyacrylamide gel electrophoresis for 2–3 h at 7.5 V/cm in a non-denaturing polyacrylamide gel with 1 × TBE buffer (0.45 M Tris base, 0.44 M boric acid, and 0.01 M EDTA, pH 8.0). The gel was then placed on Whatman filter paper, dried, and exposed to film for 1–5 days.

RESULTS

We previously described a selection strategy (see Fig. 1) for the isolation of several hepatoma variant cell lines that are specifically defective in their ability to activate the HNF4-HNF1α pathway. Despite the fact that liver-specific gene expression is rescued by the introduction of cloned HNF4 and/or HNF1α in many of hepatoma variant cell lines such as H11 and M38 [29, 31], other cell lines (e.g. M29 and HS2) could not be rescued [29].

M29 cells, as well as a panel of sister clones derived by selection against expression of the α1AT-APRT transgene, were screened for the expression of several liver-specific genes. The M29 cells failed to express 7 of the 8 liver-specific genes tested, including those expressing α1AT, β-, and γ-fibrinogen, phosphoenolpyruvate carboxykinase, albumin, argininosuccinate lyase, aldolase B and transferrin (Fig. 2a). Argininosuccinate synthetase was the exception, with low but detectable

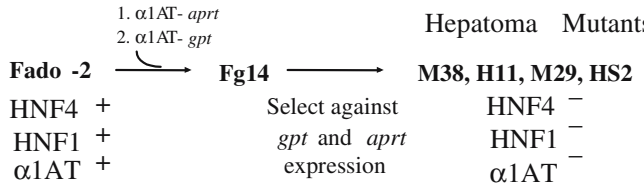

Fig. 1. Derivation of hepatoma cell lines used in this study. Plasmids pAT-*aprt* and pAT-*gpt*, containing the −640 to −2 bp α1AT promoter, were introduced into Fado-2 hepatoma cells to generate the Fg14 cell line, as described (35). Negative selection against both *aprt* and *gpt* expression was used to generate the hepatoma variant cell lines (M29, M60….). The hepatoma variant cells lack detectable expression of α1AT, HNF4 and HNF1α genes.

expression detected in the M29 cells compared to the Fg14 parental cells. Thus, the M29 cells appear to fail to express the majority of liver-specific genes.

An HNF4 expression plasmid was introduced into the M29 cells together with a plasmid encoding G418 resistance. Pooled $G418^r$ clones (>100 cells per pool) were collected and expanded. RNA was then harvested and analyzed by Northern analysis. As shown in Fig. 2b, HNF4 mRNA was absent in the M29 cells, but the introduction of an HNF4 expression plasmid produced readily detectable levels of HNF4 mRNA. We next asked whether the HNF1α and α1AT genes were activated in the HNF4 expressing M29 cells. In agreement with previous results [51], HNF4 expression restored HNF1α expression to hepatoma levels (Fig. 2c, lane 5), but failed to activate α1AT expression. The introduction of the vector alone containing the hygromycin B resistance gene into the M29 cells had no effect on expression of HNF1α or α1AT genes (lane 4). Importantly, pooled clones that survived backselection in HAT (requiring the α1AT-APRT transgene expression), resulted in full reactivation of HNF1α and α1AT expression (see "M29B", lane 16).

These results were confirmed using electrophoretic mobility shift assays (EMSA). Cell extracts were incubated with radiolabeled oligomers specific for HNF1α, HNF4, and Oct1 (Fig. 3). The Fg14 cells produced readily detectable HNF1α (left panel) and HNF4 (middle panel) binding activities. The M29 cells lack HNF1α or HNF4 protein binding (Fig. 3). The introduction of an HNF1α expression plasmid, but not the vector alone, resulted in detectable HNF1α protein binding (left panel, lane 3), but no detectable HNF4 protein binding activity. In contrast, the introduction of HNF4 into the M29 cells resulted in moderate levels of both HNF1α and HNF4 binding activity, suggesting that the HNF1α gene is activated by HNF4α expression. As expected, the M29 $APRT^+$ backselectants showed robust HNF4 and HNF1α protein binding activity. Oct1 probing was used to control for cell extract quality (right panel). Although Oct1 levels are similar in each of the cell lines, we noted that the M29 cells consistently over-express two low molecular weight proteins. The identity of these proteins is unknown, but they do not appear to correlate with expression of HNF4 or HNF1α.

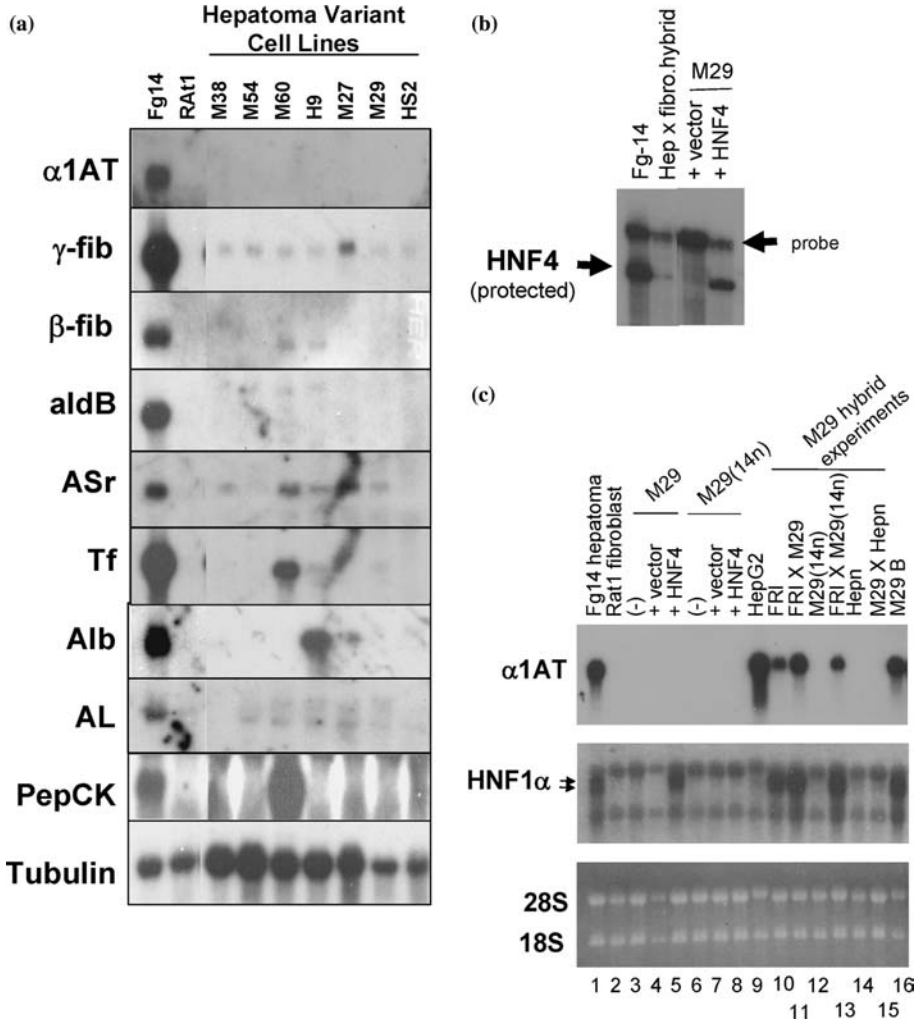

Fig. 2. HNF4 fails to rescue rat or human α1AT gene expression in M29 cells. RNA analysis of liver gene expression in M29-family cells. (a) Five micrograms of cytoplasmic RNA from indicated cell lines was size-fractionated on replicate formaldehyde gels and probed sequentially with indicated liver-specific genes. M38, M60.... are sister clones of the M29 cells described in this article. Fg14 = hepatoma parental cells. RAT1 = fibroblast cell line RNA included as a negative control. (b) RNAse protection analysis was carried out using an HNF4 antisense riboprobe. RNA from pooled M29 cells transfected with vector alone or vector + HNF4 expression plasmid were tested. A hepatoma × fibroblast hybrid cell line was included as a non-liver expressing control. (c) M29 cells (lanes 3–5) or M29(14n) cells (lanes 6–8) were stably transfected with vector alone or an HNF4 expression vector. These cell lines as well as somatic cell hybrid fusions were tested for α1AT and HNF1α expression. M29 × FRI (hepatoma) and M29(14n) × FRI cell hybrids are shown in lanes 10–13. M29 cells were also fused with an α1AT-negative liver-derived cell line, Hepn (lanes 14–15). Lastly, M29 backselectants (M29B, lane 16) were also tested. The ethidium bromide stained gel is included to verify RNA integrity. FRI = neo-marked FTO2B rat hepatoma cell line; α1AT = α1-antitrypsin; γ-fib = γ-fibrinogen; β-fib = β-fibrinogen; AldB = aldolase B; Asr = argininosuccinate synthetase; Tf = transferrin; Alb = Albumin; AL = argininosuccinate lyase; PepCK = phospho-enolpyruvate carboxykinase.

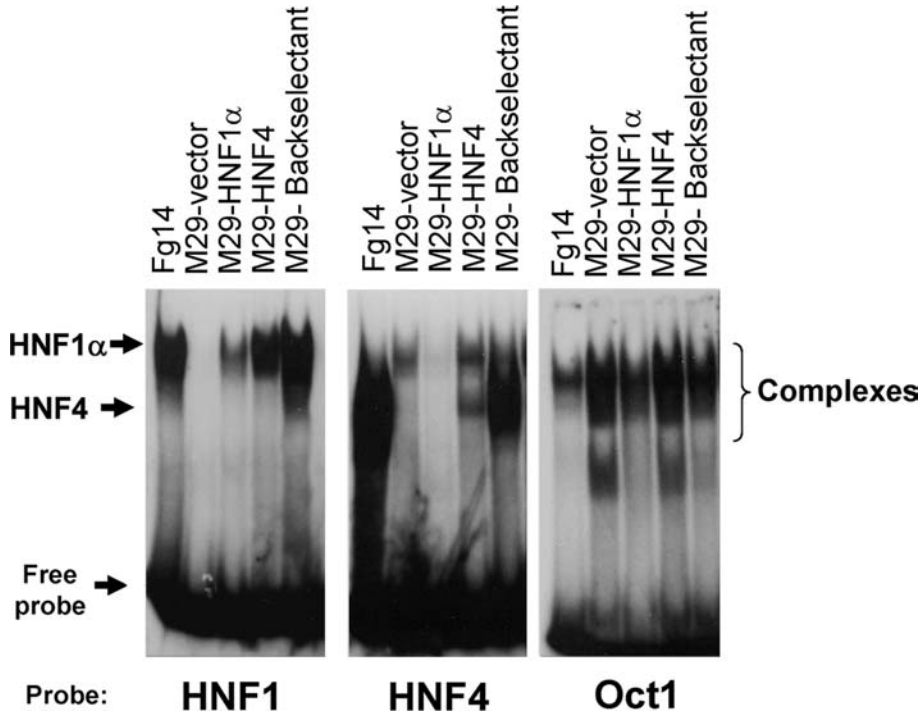

Fig. 3. Activation of endogenous HNF1α in hepatoma$^{HNF1\alpha-/HNF4-}$ cells stably transfected with HNF4. M29 cells stably transfected with HNF4 or HNF1α expression plasmids (or vector plasmid alone) were analyzed by EMSA. Nuclear extracts were incubated with end-labeled oligonucleotides specific for HNF4, HNF1α or Oct1. Protein complexes were resolved on 4% PAGE gels and exposed to film for 1–3 days. For HNF1α binding (left panel), cell extracts were first incubated at 65°C for 5 min to remove non-specific binding activity. Fg14 is the hepatoma parent of M29 cells. M29 backselectants (MB29cells) are pooled clones surviving AAT selection medium. Oct1 binding was included to verify cell extract quality.

Ectopic HNF4 and HNF1 Expression Dramatically Increases the Reversion Rate to APRT$^+$ in the M29 Cells

Because dedifferentiated cells were isolated based on the loss of their ability to drive expression of the APRT gene, counterselection in HAT or AAT allows for the isolation of GPT$^+$ or APRT$^+$ revertants, respectively. We tested the reversion rates to APRT$^+$ and GPT$^+$ in the M29 cells in the absence and presence of ectopic HNF4 or HNF1α expression (Table 1). The reversion rate to APRT$^+$ in the non-transfected cells was similar in the presence of AAT or HAT, approximately $2–5 \times 10^{-5}$. Analysis of pooled transfectants containing the G418 resistance vector alone or a HNF1α expression vector expressing a mutant form of HNF1α (designated SM, which is unable to bind DNA [37]) was unchanged (at approximately 10^{-5}). However, pooled clones expressing HNF4 or HNF1α increased reversion rates nearly 1000-fold, to approximately 1.5×10^{-2} APRT$^+$, but did not significantly change the GPT$^+$ reversion rate in HAT selection. Levels of α1AT and HNF1α RNAs in the pooled M29 backselectants were at hepatoma levels (Fig. 2c, lane 16), suggesting

Table 1. Introduction of HNF4 and HNF1α Expression Plasmids Increases $APRT^+$ Reversion Rate 1000-fold in M29 Cells. 10^7 Cells were Plated in the Indicated Selective Medium and Surviving Clones Counted at 3 Weeks using Trypan Blue Exclusion.

M29 cells	HAT	AAT
Non-transfected	5.2×10^{-5}	2×10^{-5}
Vector	$<2\times10^{-5}$	$<2\times10-5$
HNF1α	$<6\times10^{-5}$	1.3×10^{-2}
HNF1α(mutant)	$<4.3\times10^{-6}$	$<4.3\times10^{-6}$
HNF4	$<1.6\times10^{-5}$	1.6×10^{-2}

that the backselectants fully activated both the α1AT-APRT transgenes and the endogenous liver-specific genes. These results suggest that forced expression of HNF4 or HNF1α is able to activate the integrated human α1AT promoter constructs, but that the endogenous liver-specific genes are not activated to detectable levels.

The Human α1AT Locus is Silent Despite the Presence of the HNF4-HNF1α Pathway

The above results suggest that forced expression of HNF4 or HNF1α activates the human α1AT promoter. However, as shown above, ectopic HNF4 and HNF1α expression fails to activate the endogenous α1AT gene in the M29 cells. To determine whether the inability to detect α1AT gene expression is due to defective α1AT loci or differences between rat and human promoter DNA sequences, we introduced the human α1AT locus into the M29 cells. Microcell-mediated chromosome transfer was used to transfer a neo-marked human chromosome 14 from human HSF113 fibroblast cells into the M29 cells. Individual microcell hybrids were tested for the presence of the human α1AT gene using PCR analysis with specific primers for the α1AT locus. Results show that the expected size of DNA fragment was observed in several of the microcell hybrids (called M29(14n)), but not detected in pooled clones from M29 cell self fusions (Fig. 4). Therefore, the human α1AT locus was successfully transferred into the M29 cells.

We next introduced an HNF4 expression plasmid plus a hygromycin B resistance plasmid into the M29(14n) cells and pooled hygromycin B^r clones were tested for liver gene expression by Northern analysis. Human α1AT RNA was undetectable in the pooled transfectants (Figure 2c, lanes 6–8). To verify that the riboprobe used is able detect both human and rat α1AT RNA, human HepG2 hepatoblastoma cells were included (Fig. 2c, lane 9). The probe readily detected the human α1AT transcript in the HepG2 cells. Thus, these results suggest that it is unlikely that the reason that the HNF4-expressing M29 cells fail to express α1AT gene is due to a defective α1AT locus or species-related differences in promoter DNA sequences.

The Defect in the M29 Cells is Recessive

We next asked whether the defect in the M29 cells was recessive. To do this, we carried out cell fusion experiments. First, we fused the M29 cells with rat hepatoma cells marked with the neo gene (FRI cells). Hybrid cells were selected in hygromycin B + G418. Only hybrid cells were able to survive these selection conditions, as self-

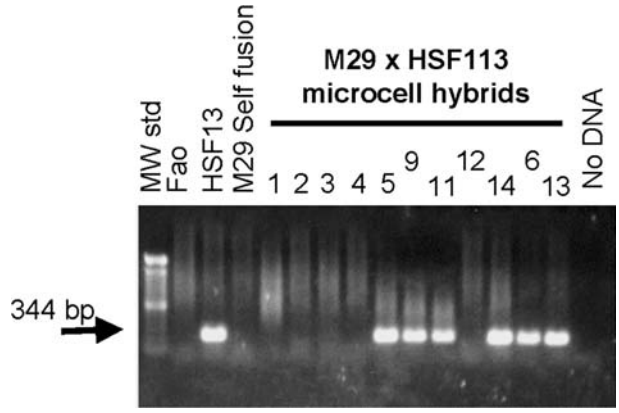

Fig. 4. Introduction of human chromosome 14 into M29 cells. Human HSF113 fibroblast cells containing a neo-marked chromosome 14 were micronucleated and chromosomes transferred into the M29 cells. Individual neo^r clones were screened for human α1AT sequences by PCR using human-specific primers predicted to amplify a 344 bp fragment. Controls include DNA from human HSF113 cells, M29 self-fusions, and no DNA included in the assay.

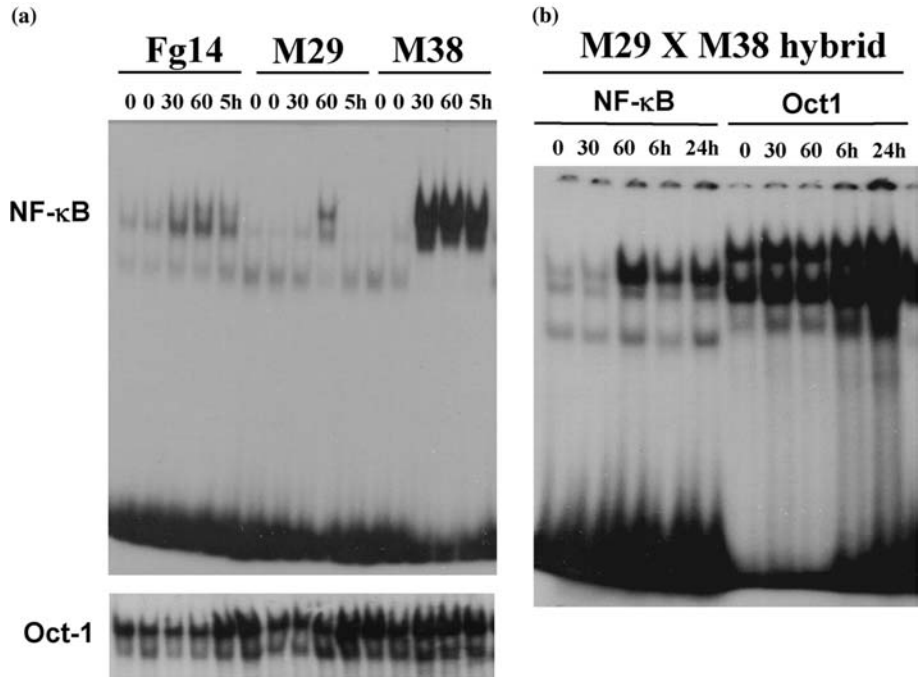

Fig. 5. NF-κB induction profile is recessive in M29 cells. Cell extracts made from Fg14, M29, M38 and M29 X M38 hybrid cells exposed to 1μg/ml LPS for 0 to 5 h were analyzed by the electrophoretic mobility shift assay (EMSA). Extracts were incubated with radiolabeled NF-κB and Oct1-specific oligomers and subjected to 4% PAGE. Gels were exposed to film for 3 days.

fusion experiments did not yield any clones. Fusion of the M29 cells with the hepatoma cells resulted in wild-type levels of α1AT expression in pooled hybrid cells (Fig. 2c, lane 13). Similar fusions were made using the M29(14n) cells, which express the introduced human α1AT locus. Fusion of the M29(14n) cells with the FRI cells resulted in wild-type levels of HNF1α and α1AT RNAs (Fig. 2c, lanes 10–12 and 13). We next asked whether fusion of the M29 cells with a hepatoma cell line (designated Hepn) that has been shown to lack α1AT expression would produce hybrids with restored α1AT expression. Results from the M29 x Hepn show that the hybrid cells fail to express α1AT or HNF1α (Fig. 2, lanes 14–15).

We further determined whether the M29 cells have a recessive defect by monitoring NF-κB binding. In agreement with previously published results [52], M29 cells produced nearly undetectable NFκB binding activity upon lipopolysaccharide (LPS) exposure, even after five h exposure (Fig. 5a). Similarly, the parental Fg14 cells showed minimal NFκB induction (Fig. 5a). Fusion of the M29 cells with another hepatoma variant cell line, M38, which has a dramatic LPS-induced NF-κB induction phenotype [52, 53], resulted in cell hybrids with an intermediate induction phenotype (Fig. 5b) that remained through the 24 h time point. As described above, the Oct 1 binding was included to verify extract quality. Thus, the cell fusion experiments suggest that the M29 cells contain a recessive defect that can be complemented by fusion with hepatoma cells.

DISCUSSION

In this report, we used a well-characterized model cell system to understand the molecular consequences of loss of tissue specific gene expression. Silencing of mammalian tissue-specific gene expression in "variant" cell lines are a well-documented epigenetic phenomenon (reviewed in [14, 15]). However, the mechanisms responsible for gene silencing in these cells are unknown. In some cases, simply the introduction of silenced transcription factors is sufficient to restore the liver phenotype (discussed below) while others are refractory to reactivation.

Here we show that the hepatoma variant cell line M29, which fails to express several liver-specific genes, does not restore α1AT gene expression with the introduction of HNF4 or HNF1α. The previously silent HNF1α gene, however, was reactivated by HNF4. Surprisingly, forced HNF1α expression failed to rescue HNF4 expression. This is in contrast to other reported hepatoma-derived cell lines described by us [29, 31] and others [30, 32] in which introduction of HNF4 or HNF1α restored expression of the other. Although the reason for this discrepancy is unclear, it is possible that the defects in the M29 cells are more profound, affecting multiple pathways involved in tissue-specific gene expression.

Despite the fact that HNF4 or HNF1α expression failed to activate downstream genes, we found that the introduction of either of these transcription factors into the M29 cells was able to increase the reversion frequency due to a selectable transgene (α1AT-APRT) by 1000-fold (from 10^{-5} to 10^{-2}). In contrast, the $HPRT^{+}$ reversion rate remained unchanged upon HNF4 or HNF1α induction. The reason for this discrepancy in unclear, since the introduced α1AT-HPRT transgene contains the same α1AT promoter sequences as the α1AT-APRT construct. It is possible that position effects are responsible for these differences, whereby the location of insertion could

affect the ability of the transgene to respond to the introduced transcription factors. However, it would be difficult to assess this possibility due to the fact that multiple copies of both constructs are integrated into the parental Fg14 cell genome [29].

Notably, the resulting frequency of reversion to $APRT^+$ was still low (10^{-2}) in the transfected cells. If only 1% of the cells have reactivated liver gene expression, this could account for the inability to detect expression of the endogenous α1AT gene. However, it was previously reported that other hepatoma variants that are rescued by introduction of HNF4 or HNF1α also show increased reversion rates due to HNF1α introduction, with the M38 cell line increasing from 10^{-6} to 10^{-1} and the H11 cells from 10^{-3} to 10^{-1} [29, 31]. Alternatively, the ability of HNF4 and HNF1α to activate the α1AT–APRT transgene without activation of the α1AT locus in the M29 cells suggest the presence of target sequences outside of the promoter sequences (640 bp) used in the α1AT-APRT construct that are targeted for repression. Species-specific differences between human and rat α1AT promoter sequences do not explain these results, as we also found that the introduced human α1AT locus contained in the M29 cells also failed to respond to HNF4 or HNF1α.

Evidence for long range regulation of the α1AT gene has been reported. Mardsen and Fournier [54] recently identified a region of DNA upstream of the human α1AT promoter sequences used in this study that is required for HNF1α-mediated modulation of chromatin in the α1AT locus. Indeed, regulation of the α1AT locus may come under distal control sequences, since a 70 kb region of the chromosome in which the α1AT locus as well as three other genes reside appear to be coordinately controlled [55].

The results using M29 cells are in contrast to other cell similar lines studied in which HNF4 or HNF1α rescues both α1AT-APRT transgene expression and α1AT gene expression [29, 56]. In the latter cases, the introduction of human chromosome 12 from a human hepatoma cell line into a hepatoma variant cell line correlated with restoration of HNF4, HNF1α, and α1AT gene expression [56].

The comparison of the M29 cells with other comparable cell lines is useful. Weiss and colleagues have isolated and characterized a series of cell lines derived from rat hepatoma cells which fail to express the liver genes, including HNF4 and HNF1α [30, 33, 57]. Three classes of cells were described, each with distinct characteristics with regard to liver expression rescue by the introduction of cloned HNF4 and /or HNF1α. Two cells lines derived from H4II cells, C5 and H5, were of particular interest. Introduction of HNF4 into the H5 cells resulted in activation of the HNF1α gene, and *vice versa*. In addition, several downstream target liver genes were also activated [24, 30]. The H5 cells were similar to cells derived in our laboratory (H11 and M38 cells) using a selection strategy in which introduction of these transacting factors rescued several liver-specific genes [29]. In contrast, the C5 cells were refractory to activation by either HNF4 or HNF1α [58]. Neither endogenous HNF4 nor HNF1α were rescued by the other in the C5 cells.

The third class of cells described by Weiss et al. were chromosomally reduced hepatoma x fibroblast hybrid cells in which the liver phenotype could be completely rescued with the introduction of either HNF4 or HNF1α [58]. This in contrast to karyotypically complete cell hybrids, which are refractory to activation by the introduction of transacting factors with the exception that HNF4 rescued HNF1α expression (but not downstream HNF1α-responsive genes) [8].

The M29 cells have a phenotype similar to cells reported by Chaya et al [58], in which a hepatoma derived cell line showed that liver gene expression appeared to be uncoupled from expression of HNF4 and HNF1α. Specifically, liver gene expression ceased despite the presence of HNF4 and HNF1α.

Mouse knockouts in the HNF4 or HNF1α have relatively unaffected RNA levels of liver gene expression, including levels of HNF4 or HNF1α mRNA. Therefore, it remains a paradox why dedifferentiated cell lines and reduced cell hybrids show such a dramatic dependence on these transcription factors for target gene expression.

Recent data using chromatin precipitation demonstrated that HNF1α and HNF4 interacts with the promoters of 100s of liver-enriched genes *in vivo*. Weiss et al also showed that certain hepatoma derived cell lines express downstream target genes in the absence on HNF4 and HNF1α, much like the transgenic HNF4 and HNF1α knock-out mice. It is unclear what accounts for these very distinct phenotypes.

In conclusion, M29 appears to be a cell line with an unusual phenotype. Liver gene expression fails to be restored upon introduction of an HNF4 expression plasmid, despite reactivation of the previously silent HNF1α gene. However, the reversion rate to $APRT^+$ is increased 1000-fold upon introduction of either HNF4 or HNF1α. The $APRT^+$ backselectants fully activated α1AT expression. It is possible that DNA methylation and/or histone modification (methylation, acetylation, phosphorylation) may contribute to the silencing of hepatic gene expression in the hepatoma variant cells. This possibility is currently being tested.

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